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TITLE: Pubertal Social Isolation and Hypervigilance Regulate Gene Expression Mechanisms of Mammary Differentiation and Cancer Risks

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14. ABSTRACT

During this grant period we demonstrated that early social isolation of rats increased the number, growth, and malignancy of naturally occurring mammary tumors developing during middle-age. Pubertal social isolation delayed mammary gland lobular involution, indicating a sensitive period for interventions that could protective against a wide spectrum of mammary pathology (from benign fibroadenomas to invasive ductal carcinomas). Early social isolation of mice accelerated SV40-T-Antigen induced tumors. In both species, social isolation during puberty induced behavioral vigilance and dysregulated the glucocorticoid response to stressors, and this predicted the development of GR+ tumors in middle-age. Finally, social isolation altered expression of 3 sets of genes in the developing mammary tissue of peripubertal mice, each in a known cancer-promoting pathway (involving fat metabolism, inflammation, and growth). During this grant period, however, the University progressively reduced our laboratory space and infrastructure, eliminating conventional rat rooms with standard caging, a specialized videography room for detailed ethograms, a hood for carcinogens, and controlled conditions for stress research. Therefore, we used different methods to achieve some of our specific aims, and could not undertake others; we therefore had an unobligated balance of $162,975. Nonetheless, we produced 9 journal articles (5 already published) and have 3 in preparation.

15. SUBJECT TERMS
Mammary cancer, mammary gland, stress, corticosterone, puberty Rat, Mouse, Spontaneous mammary tumor, gene-expression pathways

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INTRODUCTION

During Year 01 of this grant period, the Principal Investigator (PI) published her discovery that social isolation dissociates two components of puberty: it accelerates ovarian development while simultaneously delaying mammary gland development, thereby greatly increasing the exposure of developing breast parenchyma to high levels of ovarian hormones (Hermes and McClintock, 2008). In addition, socially isolated rats subsequently develop a greater mammary tumor burden during middle age, despite having entered estropause prematurely. This focused the research on puberty and young adulthood, not middle age, as a key sensitive period for increasing risk for mammary tumors. In Year 03, the PI reported in the Proceedings of the National Academy of Science that socially isolating pubertal rats increased their glucocorticoid stress response to everyday laboratory care and procedures (Hermes et al., 2009). Throughout adulthood, their glucocorticoid stress responses became progressively dysregulated and by middle age, social isolation had increased tumor malignancy, invasiveness, size and number. Importantly, these mammary tumors had receptors for glucocorticoids, the first report of stress receptors in an animal model of naturally developing breast cancer.

The ultimate goal of this grant award was to identify genes in developing mammary tissue of rats that belong to cancer-promoting metabolic pathways and are differentially expressed in socially isolated versus group-housed rodents. The PI hypothesized that these same genes would also be expressed during initiation and early growth of spontaneous mammary tumors, increasing the cancer burden of socially isolated rodents living in standard cages, even though housed in the same conventional animal laboratory room as conspecifics living together in uncrowded groups, also housed in standard caging.

Unfortunately, our rodent laboratory (accredited by and compliant with the International Association For Assessment and Accreditation of Laboratory Animal Care (AAALAC) and Office of Safety and Health Administration (OSHA)) was taken away in the beginning of the grant period. Specifically, we no longer have:

(1) Sufficient animal research and care facilities for the proposed research on rats, living in standard caging in conventional animal housing rooms, which has prevented us from conducting the proposed research utilizing our unique rat model of naturally developing mammary cancers.
(2) Access to OSHA compliant hood or facilities for using the proposed carcinogen in rats.
(3) Facilities for videography and detailed analysis of individual and social behavior in response to everyday stressors (both rats and mice).
(4) Sound isolation and proximity of wet lab facilities for tissue and sample collection, both necessary for rodent stress research.

Reinstatement of our laboratory space and research facilities became Specific Aim 5.

We took two coordinated research strategies to compensate for loss of facilities and still adhere to the ultimate goal and specific aims of this grant. Our first strategy to test the proposed hypotheses was to assay our banked rat tissue samples and reanalyze archival data sets from our rat cohorts of naturally occurring cancers, Specific Aim 2. Addressing Specific Aim 4, we determined how social isolation a) dysregulated the glucocorticoid stress response from puberty throughout the life span, b) delayed pubertal mammary development and c) the relationship between the two (Specific Aim 4, in the Revised Statement of Work). Our results refined the anatomical and physiological context in which social isolation regulates gene expression in pubertal rats, a project we will undertake once the necessary laboratory facilities are restored. This strategy was successful, yielding three publications and two completed manuscripts for peer reviewed journals ((Hermes and McClintock, 2008; Hermes et al., 2009; Pyter et al. 2009;

Yee et al. 2008; Yee et al. (submitted), Yee and McClintock (manuscript drafted)). Two were in PNAS, a high impact journal.

Our second strategy was to use FVB/N mice to address Specific Aims 1 and 3 of this grant, collaborating in our laboratory with Dr. Suzanne Conzen and Dr. Matthew Brady at the University of Chicago. Specific Aim 1 was to study gene expression in pubertal mammary tissue prior to the development of invasive cancers and Specific Aim 3 was to determine whether social isolation also accelerated vulnerability to induced mammary cancers, as it does naturally occurring tumors. We used a transgenic mouse model transfected with the S40 T-antigen (Tag), which induces invasive ductal carcinoma, rather than using the proposed DMBA, a carcinogenic chemical. Because cancer develops quickly in this mouse model, we could to simultaneously test gene expression changes in pre-malignant pubertal mammary tissue, as well as vulnerability to malignant tumor formation and growth, which occurs only weeks later. Thus we were able to combine Specific Aims 1 and 3 in a single series of experiments.

We discovered that mice deprived of social interaction from weaning exhibited increased expression of genes encoding key metabolic pathway enzymes in the premalignant pubertal mammary gland. Chronic social isolation was associated with up-regulated lipid synthesis and glycolytic pathway gene expression—both pathways are known to contribute to increased breast cancer growth. Consistent with the expression of metabolic genes in premalignant mammary tissue, isolated mice subsequently developed a significantly larger mammary gland tumor burden compared with group-housed mice. Endocrine evaluation confirmed that isolated mice developed a heightened corticosterone stress response compared with group-housed mice. This yielded two articles, (Williams et al., 2008 and Volden et al., under revision for resubmission).

The mouse project was the sole live-animal research supported by this grant. Because supplies and assay costs were funded through our Center for Interdisciplinary Health Studies Research Center, this Department of Defense grant provided essential personnel funds to support my lab manager, a data manager and a computational neuroscience graduate student, hired for just two summer months, although he oversaw the STATA program and statistical analyses throughout the grant period. They worked both on the assays and analyses of archival tissue and data sets as well as on the mouse research conducted in my laboratory. They worked on the Specific Aims and tasks itemized below. Each contributed to all reportable outcomes.

Together, these transdisciplinary studies show for the first time that an adverse social environment is associated with altered mammary gland gene expression and tumor growth, during peripubertal mammary gland development. Moreover, the identification of specific alterations in metabolic pathways gene expression favoring tumor growth suggests potential molecular biomarkers and/or targets (e.g., fatty acid synthesis) for preventive intervention in breast cancer.

BODY

Years 01-02

Accomplishments in Tasks 1 – 13. For detailed descriptions of each task, please refer to the REVISED STATEMENT OF WORK (RSOW), available in Appendix A.

Task 1. Clarify how the Revised Statement of Work (suggested in the First Annual Report Review; USAMRMC FY 06 Breast Cancer Research Program) corresponds to the original Specific Aims.

Original Specific Aims

1) Use genome wide arrays developed for the rat to identify genes differentially expressed in the mammary tissue of single and group housed rats at 60 days of age, and amplify these genes with real-time PCR to quantify their levels of expression in the two social environments.

2) With the same methods, determine whether rats with a hypervigilant personality differentially express the same set of genes, with additive effects in socially isolated animals or in rejected animals within a social group.

3) Determine whether social isolation and hypervigilance increase susceptibility to the carcinogen, DMBA, administered at the same age and stage of mammary differentiation.

Revised Specific Aims

Specific Aim 1 remained the same, except we substituted FVB/N mice to identify the genes differentially expressed peripubertally in socially isolated and group housed rodents, rather than out bred Sprague-Dawley rats. We worked in my laboratory in collaboration with Prof. Suzanne Conzen, M.D., a molecular oncologist in the Department of Medicine also at the University of Chicago and Prof. Matthew Brady, Ph.D., an endocrinologist also in the Department of Medicine. We studied gene expression prior to the onset of invasive cancer in this C3 (1)/SV40 T-antigen (Tag) FVB/N transgenic mouse model of breast cancer.

We were concerned that we might not replicate our findings in mice living in partial filter top caging, which prevent pheromonal communication within our animal colony. Fortunately, the mouse's response to pheromonal isolation is opposite that of rats, and we replicated our rat findings (William et al., 2009), enabling the substitution required by our lack of a rat laboratory with conventional housing in standard cages, conditions of the foundational body of research on which the proposed research is based.

Specific Aim 2. We could not complete this aim because our specialized behavioral videography and testing room was reassigned to other faculty, preventing our planned analysis of subtle emotional reactions and complex social interactions within the groups (such as reciprocal affiliation during a stressor, Yee et al., 2008). Instead we modified a colony room and tested mice for vigilance and anxiety by direct observation in a small exploration stress arena, but without benefit of videography and a full ethogram, working around the lack of isolation from uncontrolled stressful noises from the hallways. An adjacent colony room served as a wet lab.

Specific Aim 3. Access to a hood or biosafety cabinet that met safety regulations for carcinogens was not restored to us within the grant period, and so we could not use the proposed carcinogens NMU or DMBA to induce and accelerate mammary cancer development. We replaced our standard DMBA-induced rat mammary cancer model with the C3 (1)/SV40 T-antigen (Tag) transgenic mouse model. In this transgenic mouse, the cancer-promoting agent is the Simian Vacillating Virus 40 large T antigen, which is a proto-oncogene and also inactivates tumor suppressor genes such as p53. Transgenic female FVB/N mice are homozygous for the SV40 Tag gene, driven by a fragment of the promoter of the gene encoding the rat prostatic steroid binding protein. The mice develop mammary hyperplasia at 11 weeks of age, during pubertal mammary development, noninvasive tumors at 15 weeks of age in young adulthood, and all have invasive mammary cancers by 22 weeks of age, in mid-adulthood. The SV40 T-antigen promoter is not sensitive to glucocorticoids, and so any difference between socially isolated and group housed mice cannot be attributable to transgenic status.

Specific Aim 4 (Added in the RSOW).

We formalized Specific Aim 4 when it became clear that our rat laboratory, facilities, and equipment would not be restored. In place of rats, we used our considerable bank of stored rat tissue and data to continue rat research, such as responding to a reviewer's request that we diagnose each tumor. This was successfully published in the high-impact factor journal, Proceedings of the National Academy of Sciences (Hermes et al., 2009).

A major accomplishment was histochemical clinical diagnosis and receptor status of tumor type, conducted in collaboration with Bertha Delgado, M. D., a breast pathologist at Be'er Sheva University. This work supported our hypothesis that social isolation not only increases tumor burden, but also malignancy and tumor progression (Hermes et al., 2008, Hermes et al., 2009, Williams et al., 2009). Using archival data is our best solution, until our rat laboratory is restored. In addition, we undertook assaying and analyzing archived tissue, completed the results and have outlined two manuscripts for submission to anatomy journals on: (1) development of the rat mammary gland and (2) typology or rat tumors. Each underscore fundamental aspects of rat mammary gland biology of spontaneous breast cancer as well as its developmental origins.

Specific Aim 5 (Added in the RSOW). Continue efforts to recover our lost research space and infrastructure, needed for this grant's mouse and rat research, and reopen our rat laboratory. The four functions needed to resume the original statement of work are:

(1) a rat laboratory for conventional housing in standard caging
(2) facilities for sound-isolated videography and data collection for detailed analysis of individual and social behavior in response to everyday stressors
(3) sound isolation and proximity of animal housing to testing rooms and wet labs for blood sample and tissue collection and necropsy, both necessary for reliable stress research and gene expression experiments
(4) facilities and equipment for use of carcinogens meeting OSHA standards

Task 2. Behavioral determination of newborn temperament and stress vulnerability

In our former rodent videography and behavioral testing room, we could quantify a temperament continuum from vigilant to bold. In our modified mouse exploration stress arena, without video recording of the full ethogram, we could only quantify latency to leave home base as a measure

of vigilance. Nonetheless, we could use this single measure to constitute two cohorts of socially isolated and group-housed mice, balanced for vigilant temperament, and to establish that mice assigned to social isolation become vigilant (Williams et al., 2009).

This single behavioral measure was not robust enough, however, to enable testing our hypothesis that rodents born with a vigilant temperament have the same pattern of mammary gene expression as mice that became vigilant from imposed social isolation (Original Specific Aim 2).

Task 3. Measuring individual differences in estrous cycle activity

We used vaginal cytology in the daily vaginal lavage to balance cohorts for ovarian cyclicity and estrogenization, whenever we needed to constitute two cohorts, and assign one to imposed social isolation. We also used vaginal cytology to euthanize and harvest tissue from rodents all at the same phase of the cycle and estrogen level (Hermes et al., 2008, Hermes et al., 2009, Williams 2009, Volden et al., manuscript under revision, Yee et al., 2008, Yee et al., manuscript for resubmission)

Task 4. Assess corticosterone reactivity to a mild stressor

Socially isolated mice have dysregulated corticosterone stress responses, indistinguishable from those of Sprague-Dawley rats. Corticosterone rises to higher levels, and recovers more slowly. Social isolates also develop larger mammary cancers earlier than their group housed counterparts, and so are correlated in this sense. More importantly, within groups of rats, those individuals that are more stress reactive are more likely to develop large tumors more quickly (Yee et al., under revision for resubmission).

Task 5. Repeat behavioral tests at 13 weeks of age, young adulthood.

As with newborn temperament, we did not have the videography room necessary for data collection of detailed ethograms of stress reactivity, personality and social interactions. Our measure of latency to leave home base, however revealed temperament stability throughout the life span (Hermes et al., 2009, Williams et al., 2009).

Task 6. Dissect ovaries and mammary glands of each female, staining, and receptor status

All immunohistochemistry, whole mounts for mammary gland development, tumor diagnosis and receptor status were excellent and reported in the accompanying articles (Hermes et al., 2008, Hermes et al., 2009, Williams et al., 2009, Pyter et al., 2009, Yee et al., 2008, Yee, under revision for resubmission, Yee et al., manuscript in preparation)

Task 7. Tissue Bank and Databank on server.

These full necropsy tasks were dropped because mice have a small body size. In place, we had planned to focus on behavioral video analyses, described above in Task 2, but our behavior videography room was no longer available to us. We did analyze banked rat tissue for the above manuscripts.

Task 8. Identification of pattern of differential gene expression in mammary glands harvested from socially isolated and group housed mice.

Because cancer development is a multistep process, we examined gene expression changes in mammary glands from isolated versus group-housed mice at 15 weeks of age (late puberty and early adulthood) when the mammary gland is still differentiating and any hyperplasia is not yet invasive cancer. We hypothesized that differences in the social environment might be associated with altered gene expression in the mammary glands because the social environment has been previously linked to significant gene expression changes in various tissues, including the central nervous system of mice and the peripheral blood lymphocytes of humans. To examine gene expression differences, RNA was extracted from a subset of 15-week-old mammary glands (n = 4 mice in each group) all without invasive cancer.

We next identified specific functional and disease categories using IPA12 for the differentially expressed genes from isolated versus group-housed mice. Among the 15-week-old mice, we found that gene expression related to immunologic disease (P = 4.08E−09), inflammatory disease (P = 8.67E−09), and lipid metabolism (P = 2.73E−08) was significantly different in the isolated versus group-housed mouse mammary glands (Williams et al., 2009; Volden et al., under revision for resubmission).

Finding that social isolation exacerbates inflammatory function within mammary tumors complements our discovery that rat mammary tumors themselves secrete cytokines, which in turn increase depression and anxiety (Pyter et al., 2009). Thus, once a female rodent develops a mammary tumor she enters a “feed-forward” system whereby stressors and anxiety accelerate tumor growth, and in turn tumors exacerbate anxiety and depression.

Among the metabolic genes, significantly increased gene expression was identified in genes encoding three key enzymes known to be associated with cancer development: ATP citrate lyase (Acly), acetyl-CoA carboxylase cancer or palpable tumors. Global steady-state mRNA levels were measured using Affymetrixte technology and the Robust Multiarray Average (RMA) algorithm (13) was used for normalization. Using a false discovery rate of 5% and a minimum fold change of ≥1.25 (≤0.80 for down-regulation), 296 downregulated and 68 up-regulated transcripts were identified in 15-week-old mouse mammary glands.

We similarly examined mammary gland gene expression in mammary glands from mice that were 20 weeks old that contained no detectable carboxylase α (Acaca), and hexokinase 2 (Hk2). The human orthologues of mouse Acly and Acaca were previously shown to be upregulated in aggressive, metastatic breast cancer cell lines and to be essential for breast cancer cell survival, respectively. We therefore examined whether there was an increased expression of mouse Acly, Acaca, and Hk2 mRNA by quantitative RT-PCR using individual mammary gland RNA from socially isolated versus grouped housed mice.

This analysis confirmed a 1.5- to 3-fold increase in steady-state mRNA expression in mammary glands from isolated versus group-housed mice for all three metabolic pathway genes, suggesting that chronically isolated transgenic mice exhibit gene expression changes associated with increased glycolytic and lipogenic metabolic pathway activation. Interestingly, activation of these pathways has been postulated to be a precursor to tumor development in several cancer model tumors.

Individual gene expression was validated. Quantitative RT-PCR values are given as fold change relative to expression in group-housed mice (n = 4 individual mice per cohort). Differences in expression were found to be significant based on the ΔΔCt method estimates using a mixed-effects ANOVA model (Error bars = SEM, Acaca, P = 0.0005; Acly, P < 0.0001; Hk2, P < 0.0001). The top functional gene categories associated with social isolation based on mammary

gland gene expression differences at 15 wk were identified using the IPA 6.5 software. These were: human HK2 (hexokinase 2), ACL (ATP citrate lyase), and ACC (acetyl-CoA carboxylase α). All three genes encode key components of cancer-associated glycolysis and lipogenesis pathways.

Task 9. Psychosocial effects on susceptibility to SV40 large T-antigen-induced mammary cancer.

The same histological techniques were used for mouse and archived rat tumors. Paraffin-embedded mouse mammary gland and tumor sections were assessed for differentiation using a Bloom and Richardson System that is based on architectural features (i.e. extent of tubule formation). For example, in Williams (et al., 2009), a total of 52 tumors from eight group-housed and 62 tumors from nine isolated mice were evaluated. Invasive mammary adenocarcinomas showing tubular (i.e. glandular) or papillary formation in more than 75% of the tumor will be classified as grade 1 (well-differentiated). Tumors in which tubular formation constituted between 10 and 75% will be classified as grade 2 (moderately-differentiated), and tumors exhibiting less than 10% tubular formation will be classified as grade 3 (poorly-differentiated).

Task 10. Mammary gland differentiation and tumor identification

H and E analysis and histopathology were employed to assess rat's mammary tissue differentiation and diagnose the full spectrum of their spontaneously developing tumors. Microarrays of tumor tissue were constructed and assayed for multiple steroid receptors: ER, PR, and GR, dramatically cutting costs (Hermes et al. 2009, Williams et al., 2009, Yee et al., under revision for resubmission).

Task 11. Multivariable matrix-based database established and analyzed in STATA.

Identified specific functional and disease categories using IPA12 for the differentially expressed genes from isolated versus group-housed mice (Williams et al., 2009) During the grant period we consolidated many of our databases and statistical analysis to STATA, transitioning from a set of diverse statistical packages (Statview, SPSS, SAA and Excel), each of which is based on "flat", not matrix-based, data sets. We tested hypotheses with diverse statistical methods: multivariate modeling, log survivor analysis, logistic regression and analysis of variance. We continue to work toward computational models for the synergistic interactions of interdependent biological systems, operating across levels of analysis: social, psychological, endocrine and metabolic, cellular and genetic.

Task 12. Submit high priority PNAS manuscript based on Specific Aim 4

We completed the tumor diagnosis utilizing archived tissue, as requested by the review process of an earlier version, based only on tumor size and number, as well as additional analyses from archived data sets. The new manuscript was submitted to PNAS.

Task 13. Reinstate conventional rat laboratory for biopsychological research.

Both the PI and staff have spent considerable time continuing our efforts to reopen our laboratory, currently missing four key elements: (1) a conventional rat laboratory for standard caging, (2) sound isolation and proximity of facilities for tissue and sample collection, both necessary for stress research (3) facilities and equipment for use of carcinogens meeting OSHA standards and (4) facilities for videography and detailed analysis of individual and social

behavior in response to everyday stressors. The missing the last three has also hampered our research with transgenic mice. This was not accomplished.

Year 03

Task 14. Set 2 replicating and extending Set 1.

We ran several sets of mice (Sets 1 – 3) and verified that social isolation in Sets 2 and 3 regulates gene expression in the same three metabolic pathways identified in Set 1.

Task 15. Resume both the original and revised statement of work in restored rat conventional laboratory with standard caging, to study the effects of social isolation on gene regulation, when rats are living under the same social, pheromonal and environmental conditions used in our longstanding research program. These are the conditions in which had repeatedly demonstrated that social isolation increases the number, size and malignancy of spontaneously developing mammary cancers in rats.

In addition, while preparing for the experiments to identify genes in mammary tissue, the PI made a very informative discovery. When the PI added the proposed measures of individual differences in a vigilant newborn temperament, the PI found that the isolates had accelerated mammary development. The working hypothesis is that the early experience of mild stressors and calming handling changed the developmental trajectory of isolated rodents. The PI monitored members of the same cohort until middle age to in an effort to establish whether the isolates under these conditions (mild stressors vs. calm environment) are also protected from mammary tumors.

In a separate cohort that was not given any additional experiences in the neonatal and pre-weaning period, the findings were consistent with previous studies, documenting early onset of mammary tumors in socially isolated animals. The PI found that social isolation dissociated two components of puberty: it delayed mammary tissue development while it simultaneously accelerated maturation of ovarian function, thereby increasing the exposure of developing breast parenchyma to high levels of estrogen. Concomitantly isolated females in this cohort went on to develop a larger mammary tumor burden than rodents living in groups.

If the reversed pattern of mammary development is also associated with a greater tumor burden, the PI will have a stronger model to identify not only the gene expression mechanisms underlying increased risk in social isolation, but also genetic mechanisms mediating the positive effects of a punctate early psychosocial and hormonal intervention that ameliorates risk in a population highly vulnerable for mammary tumors.

This task was not accomplished. Work could neither be resumed on this discovery, nor the rat gene expression studies conducted, because the rat laboratory facilities and equipment did not become available before the end of the grant period. An unobligated balance of $162, 975 remained at the end of the grant period.

Task 16. Publish journal articles and prepare manuscripts for submission.

The 9 published and submitted journal articles, as well as results of 3 manuscripts in preparation for submission, are listed under reportable outcomes, and available in Appendix B. Many more will continue to flow, and are in various stages of completion, ranging from figures, to outlines and rough drafts. This grant will be acknowledged in each.

KEY RESEARCH ACCOMPLISHMENTS

• Social Isolation regulates genes in three distinct metabolic pathways in the mammary gland: lipogenesis, glycolysis and inflammation. Each of these genes promotes cancer cell proliferation, which takes energy.

•There are several plausible “downward causation” pathways enabling the social world to “get under the skin” and regulate genes known to accelerate cancer.

• Identified an early psychosocial intervention, experience of mild stressors along with calming handling, changes the developmental trajectory of mammary tissue sustained during young adulthood, between 2 and 6 months of age.
• Identified 20 days of age, preweaning, as a sensitive period for these enduring effects.
• Identified lobular involution as the developmental process affected by the intervention.

• Identified an early psychosocial intervention, experience of mild stressors along with calming handling, changes the developmental trajectory of mammary tissue sustained during young adulthood, between 2 and 6 months of age and reduces the number, multiplicity and total burden of mammary tumors that developed spontaneously by early middle age.

• Identified 20 days of age, preweaning, as a sensitive period for these enduring effects, and demonstrating the primacy of post pubertal social conditions over inborn temperament.

• Identified lobular involution as the developmental process affected by the intervention and linked it to tumor risk.

Importantly, these mammary tumors had receptors for glucocorticoids, the first report of stress receptors in an animal model of naturally developing breast cancer

Together, these transdisciplinary studies show for the first time that an adverse social environment is associated with altered mammary gland gene expression and tumor growth, during the time of mammary development. Moreover, the identification of specific alterations in metabolic pathways gene expression favoring tumor growth suggests potential molecular biomarkers and/or targets (e.g., fatty acid synthesis) for preventive intervention in breast cancer.

REPORTABLE OUTCOMES

Funded grant based on DoD research.

Identifying mechanisms linking stress biology to human breast cancer.
1R01CA148814-0141
The National Cancer Institute
Suzanne D. Conzen, MD PI
Martha K. McClintock, PHD PI
06/20/2011 – 04/30/2016

12 Publications and Manuscripts

Published

1. Hermes GL, Delgado B, Tretiakova M, Cavigelli SA, Krausz T, Conzen SD, McClintock MK. Social isolation dysregulates endocrine and behavioral stress responses while increasing the malignant burden of spontaneous mammary tumors. **Proceedings of the National Academy of Sciences, USA** 106:22393- 22398, 2009. PMC2799783
2. Hermes, G.L., McClintock, M.K. Isolation and the timing of mammary gland development, gonadarche, and estropause: implications for mammary tumor burden. **Developmental Psychobiology**. 50, (4): 353-360, 2008.
3. Pyter LM, Pineros V, Galang JA, McClintock MK, Prendergast BJ. Peripheral tumors induce depressive-like behaviors and cytokine production and alter hypothalamic-pituitary-adrenal axis regulation. **Proceedings of the National Academy of Sciences** USA 106:9069-9074, 2009. PMC2689998
4. Williams JB, Pang D, Delgado B, Kocherginsky M, Tretiakova M, Krausz T, Pan D, He J, McClintock MK, Conzen SD. A model of gene-environment interaction reveals altered mammary gland gene expression and increased tumor growth following social isolation. **Cancer Prevention Research (Phila Pa)** 2:850-861, 2009.
5. Yee JR, Cavigelli SA, Delgado B, McClintock MK. Reciprocal affiliation among adolescent rats during a mild group stressor predicts mammary tumors and life span. **Psychosomatic Medicine** 70:1050-1059, 2008.

Submitted, Revision In Process

6. Volden PA, Wonder EL, Skor MN, Carmean CE, Ye H, Kocherginsky M, Eleanor Smith1, Kregel S, McClintock MK, Brady MJ, and Conzen SD. Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue. **Endocrinology**.
7. Yee JR, Cavigelli SA, Delgado B, Krausz T, Conzen SD, McClintock MK. Individual Variation In Stress-Induced Corticosterone Dynamics And Spontaneous Mammary tumor Risk. **Psychoneuroendocrinology**.
8. Yee JR and McClintock MK. Psychosocial modulation of mammary gland involution and spontaneous mammary tumor risk. **Developmental Psychobiology**.

Submitted

9. Gehlert S, Obeid E, Bollinger S, Conzen S, McClintock MK Polite B, Olopade O Pathways by Which Social Environment Affects Biology to Produce Health Disparities. Invited manuscript for **Health Affairs**

Manuscripts (Results Completed)

10. Hermes G, McClintock MK. Foraging Strategies: Development of Cognitive Strategies in a Spatial Task: Endocrine & Psychosocial Mediators. Manuscript ready for submission to **Learning and Motivation**

11. Yee JR Delgado B, Krausz T and McClintock MK. Redefining Puberty: Lobular Involution Of The Mammary Gland. **Anatomical Record**.

12. Yee JR, Delgado B, Krausz T, McClintock MK. Spontaneous Mammary Tumors In Norway Rats: A Natural Model for Human Breast Cancer and Benign Pathology. **Modern Pathology**

CONCLUSIONS

- Early social isolation of rats increases the number, growth, and malignancy of naturally occurring mammary tumors developing during middle age.

- Early social isolation of mice accelerated growth of tumors induced by the SV40-T-Antigen.

- Pubertal social isolation delayed mammary gland lobular involution in rats, indicating a sensitive period for interventions that could protective against a wide spectrum of mammary pathology (from benign fibroadenomas to invasive ductal carcinomas).

- In the mouse model, social isolation altered expression of 3 sets of genes in the developing mammary tissue of peripubertal mice, each in a known cancer-promoting pathway (involving fat metabolism, inflammation, and growth).

- In both rats and mice, social isolation during puberty induced life long behavioral vigilance and dysregulated the glucocorticoid response to stressors, and this predicted the spontaneous development of GR+ tumors, both benign and malignant, in middle-age rats, and the accelerated growth of invasive ductal carcinomas inevitably induced by the SV40-T-Antigen.

Together, these transdisciplinary studies show for the first time that an adverse social environment is associated with altered mammary gland gene expression and tumor growth, during the time of mammary development. Moreover, the identification of specific alterations in metabolic pathways gene expression favoring tumor growth suggests potential molecular biomarkers and/or targets (e.g., fatty acid synthesis) for preventive intervention in breast cancer.

REFERENCES

Not Applicable

APPENDICES

Appendix A. Revised Statement of Work
Appendix B. Publications and Manuscripts

APPENDIX A

MCCLINTOCK, MARTHA K.

Effect of Pubertal Social Isolation and Hypervigilance on Gene Expression
In Differentiating Mammary Tissue and Susceptibility to a Carcinogen

Revised Statement of Work for
Year 02 (2008-2009) and Year 03 (2009-2010)

Year 02

Task 1. Clarify how this Revised Statement of Work (which was suggested by the First Annual Report Review Year 01; USAMRMC FY 06 Breast Cancer Research Program) corresponds to the Original Specific Aims.

Original Specific Aims

We aim to:

1) Use genome wide arrays developed for the rat to identify genes differentially expressed in the mammary tissue of socially isolated and group-housed rats at 60 days of age, and amplify these genes with real-time PCR to quantify their levels of expression in the two social environments.

2) With the same methods, determine whether rats with a hypervigilant personality differentially express the same set of genes, with additive effects, in socially isolated animals or in rejected animals within a social group.

3) Determine whether social isolation and hypervigilance increase susceptibility to the carcinogen, DMBA, administered at the same age and stage of mammary differentiation.

Revised Specific Aims

Specific Aim 1 will remain the same, except we will use the SV40 T-antigen (Tag) FVB/N transgenic mouse model of breast cancer to induce mammary cancer in pubertal animals, instead of inducing them in rats with the carcinogens DMBA and NMU. We will still identify the genes differentially expressed in mammary tissue of socially isolated and group housed transgenic mice, in place of Sprague-Dawley rats that develop mammary cancers naturally. This will be accomplished by collaborating in my laboratory with Prof. Suzanne Conzen, M.D., a molecular oncologist in the Department of Medicine also at the University of Chicago.

Specific Aim 2 can no longer be completed because our specialized behavioral videography and testing room has been reassigned to other faculty, preventing our planned analysis of subtle emotional reactions and complex social interactions within the groups (such as reciprocal affiliation during a stressor. We will modify a standard colony room and test mice without videography, measuring vigilance and anxiety in a small exploration stress arena, and scoring it by hand in real time.

Specific Aim 3 is modified because we no longer have access to a hood or biosafety cabinet safe for using carcinogens, precluding use of the proposed carcinogen DMBA or NMU to stimulate mammary cancer. We will replace our standard DMBA-induced rat mammary cancer model with C3 (1)/SV40 T-antigen (Tag) FVB/N mice, collaborating in my laboratory with Prof. Suzanne Conzen, M.D., a molecular oncologist in the Department of Medicine also at the University of Chicago. Thus, we will consolidate experiments for our original and revised Specific Aims 1 and 3 into a single series of experiments.

First, we will test the hypothesis that social isolation increases vulnerability to virally induced mammary cancer (Specific Aim 3 above). Second, in subsequent experiments, we will measure patterns of differential gene expression in mammary glands of pubertal and young adult mice **prior to development of invasive cancers** (Specific Aim 1 above).

Detailed Rationale for Modifying Specific Aim 3**.** In this transgenic mouse, the cancer-promoting agent is the Simian Vacillating Virus 40 large T antigen, which is a proto-oncogene and also inactivates tumor suppressor genes such as p53. Transgenic female FVB/N mice are homozygous for the SV40 Tag gene, driven by a fragment of the promoter of the gene encoding the rat prostatic steroid binding protein. The mice develop mammary hyperplasia at 11 weeks of age, during pubertal mammary development, noninvasive tumors at 15 weeks of age in young adulthood, and all have invasive mammary cancers by 22 weeks of age, in mid-adulthood. The SV40 large T-antigen promoter is not driven by glucocorticoids, and so any difference between socially isolated and group housed mice can not be attributable directly to their transgenic status.

The SV40 T-antigen promoter is not sensitive to glucocorticoids, and so any difference between socially isolated and group housed mice would not be an artifact of their transgenic status. Working in my laboratory in collaboration with Dr. Suzanne Conzen of the Department of Medicine at the University of Chicago, our combined labs demonstrated that socially isolated mice also have dysregulated corticosterone stress responses, as do our Sprague-Dawley Rat model, they also develop larger mammary cancers earlier than their group housed counterparts. We could obtain these results housing mice in filter top caging, circumventing our lack of conventional housing in standard cages, which has been the foundation of our previous research program described in the proposal.

This mouse project will be the sole live-animal research supported by this grant, until restoration of the facilities, resources and equipment needed for our standard rat laboratory and rodent stress research. Specific gene expression changes will be more readily detected against the genetically homogeneous background of our transgenic mouse model. This approach will dramatically increase our power to detect socially regulated gene expression changes in the genetically heterogeneous out bred-rat model of spontaneous mammary cancers. Informed by our transgenic mouse model, we can target specific gene regulation within our rat model of mammary gland development and concomitant increase in spontaneous mammary tumor burden.

Two Additional Specific Aims

Specific Aim 4

We will utilize our considerable bank of stored rat tissue and rat data to respond to reviewer's critiques of our manuscript submitted to the Proceedings of the National Academy of Science, including their request for mammary tumor diagnosis and steroid

receptor status. The major advance will be histochemical analysis of the tumors to determine which are benign and malignant. Our specific hypothesis is that social isolation not only increases tumor burden (number and growth rate), but that it also increases malignancy and tumor progression. Using archival data is our best solution, until our rat laboratory is restored. In addition, we will undertake archival analyses to prepare for publications on the development of the rat mammary gland and the typology or rat tumors, both fundamental aspects of mammary gland and tumor biology in our rat model of spontaneous breast cancer. This will provide a stronger foundation for targeting our research when we resume our rat research.

We will collaborate with Bertha Delgado, M.D., a breast pathologist from Be'er Sheva University in Israel, on sabbatical in the Department of Pathology at the University of Chicago. She will clinically diagnose and assess receptor status of each mammary tumor. Using archival data and tissue is our best solution, until our rat laboratory is restored.

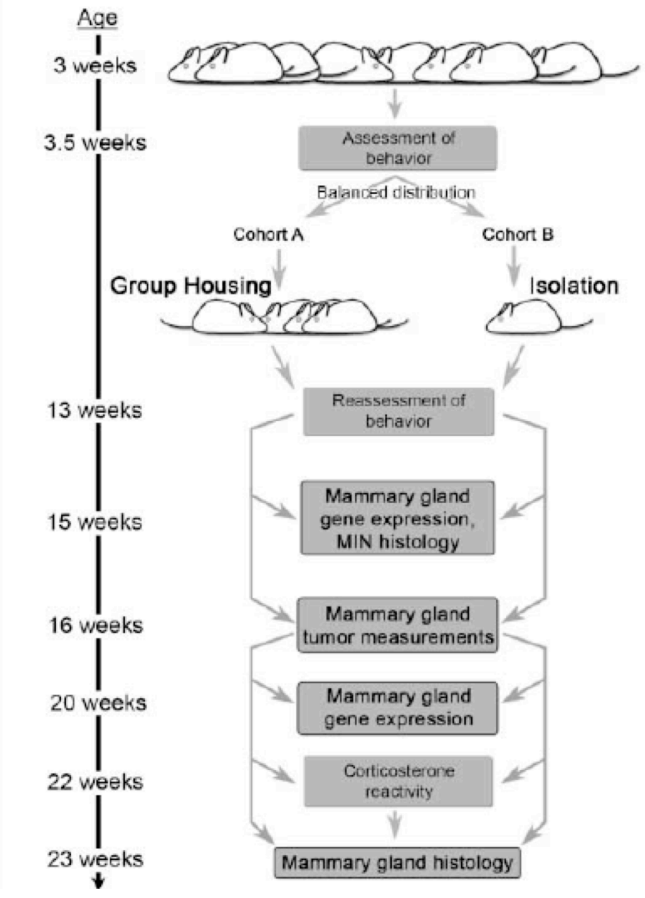
Specific Aim 5

We will continue our efforts to recover our lost research space and infrastructure and reopen our rat laboratory. The four functions needed to resume the original statement of work are:

(1) a rat laboratory for conventional housing in standard caging
(2) facilities for sound-isolated videography and data collection for detailed analysis of individual and social behavior in response to everyday stressors
(3) sound isolation and proximity of animal housing, testing rooms and necropsy for blood sample and tissue collection, both necessary for reliable stress research and gene expression experiments
(4) facilities and equipment for use of carcinogens meeting OSHA standards

Task 2. Behavioral determination of individual's temperament and stress vulnerability

a.) ACUP submitted for IACUC review and approval.
b.) Breed C3 (1)/SV40 T-antigen (Tag) FVB/N mice in the viaticum of Prof. Suzanne Conzen, M. D., Department of Medicine, University of Chicago.
c.) Age 3.5 weeks of age, perform Exploration Stressor Test to ascertain hypervigilance to new environments and thereby vulnerability to stressors. Employ videography to identify not just activity, but species-typic exploration and foraging strategies, as well as behaviors



indicating

d.) Constitute Set I: Cohort A: 4 mice living together in one group and Cohort B 4 socially isolated rats in single cages, matched for temperament and family.

Task 3. Measuring individual differences in estrous cycle activity.

a.) Daily observation for onset of vaginal patency, first stage of pubertal process.
b.) Analysis of daily vaginal cytology as a bioassay of estrogen and progesterone levels, and age when regular estrous cycles are established, another stage of pubertal process.
c.) Weekly nipple development measurements as a biomarker for mammary tissue differentiation, a key part of pubertal process.
d.) Euthanization and tissue harvest at lights-on (dawn) of the day when the majority of group housed females are estrus.

Task 4. Assess corticosterone reactivity to a mild stressor.

a.) Twenty-two week old mice will be gently restrained in a tube simulating a collapsed burrow runway. Animals were restrained within a ventilated 50mL conical tube for 30 minutes. Blood samples will be collected using the tail-nick method at baseline and 30, 60, and 120 min following initiation of the restraint. Whole blood (30μL) will be collected at each time point, and following centrifugation, serum was stored at -20°C.
b.) The corticosterone assay will be carried out using a double antibody radioimmunoassay kit (MP Biomedicals).

Task 5. Repeat behavioral tests at 13 weeks of age, young adulthood.

a.) Ascertain with videography each animal's ability to individually or socially buffer stress responses to a novel environment and to everyday husbandry activities.
b.) Videography will also be essential to record for later detailed analysis the social interactions indicating reciprocity of care, a social role known to predict high resilience to mortality from mammary cancer in rats.
c.) Daily observations of home cage behavior, during animal care intrusions, and when alone, as indicators of metabolic activity and thermoregulation.

Task 6. Dissect mammary glands of each female, staining, and receptor status.

a.) Pectoral mammary glands #3 and #4 and Inguinal mammary glands #9, #10: counterbalance assignment of one side for gene array plus real time PCR, and the other for real time PCR verification. (See Task 7 below).
b.) Pectoral and inguinal mammary glands from 10.5, 15, and 22-week-old mice will be harvested immediately following sacrifice by CO2 inhalation. Following rapid dissection, the five right lateral mammary glands will be immediately wrapped in aluminum foil and flash-frozen in liquid nitrogen. The glands will be stored at -80 °C for later RNA, DNA and protein extraction. Left lateral mammary glands will be dissected next and immediately formalin-fixed, followed by paraffin embedding within 24 hours. Paraffin-embedded 4μm sections will be stained for hematoxylin and eosin (H&E) and immunohistochemistry. Following antibody optimization, paraffin sections were prepared as described previously (Sahoo et. al, 2005) and stained with the following rabbit polyclonal antibodies: Anti-Estrogen Receptor α (MC-20, 1:100, Santa Cruz Biotechnology), anti-Progesterone Receptor (Ab-13, 1:200, Lab Vision Corporation), anti-Glucocorticoid Receptor (M-20, 1:200, Santa Cruz Biotechnology), anti-Ki67 (SP-6,

1:200, Lab Vision Corporation), and anti-cleaved Caspase-3 (CP229A, 1:50, Biocare Medical) antibodies.

Task 7. Tissue Bank: (for future measures). Establish Databank on server for Tissue Bank.
This specific task will be dropped, given the small body size of mice. Rather we will focus on behavioral and video analyses, described above in Task 3.

Task 8. Identification of pattern of differential gene expression.

a.) Total RNA was isolated from flash frozen right pectoral mammary glands from standard-housed and isolated mice (n = 4 each) using the RNeasy Mini Kit (Qiagen). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies), and concentration and 260/280 ratios (> 1.8 for each sample will be considered acceptable) were determined using an ND-1000 spectrophotometer (NanoDrop). The Mouse Genome 2.0 Array GeneChip (Affymetrix) was used for gene expression analysis.
b.) Gene expression in the standard-housed and isolated 15 week-old mice was analyzed. Data were first normalized using MAS 5.0 (Affymetrix), and genes with at least one absent call (as determined by MAS 5.0) in the group with higher average expression were excluded. Normalization was performed using the "affy" package in Bioconductor (http://www.bioconductor.org).
c.) Differences in expression between isolated and standard-housed animals will be assessed using Significance Analysis of Microarrays (SAM). In SAM, each gene is assigned a score di, which is proportional to the difference in expression between the two groups relative to the standard deviation, and is similar to the t-statistic. Genes with scores exceeding adjustable cut-offs are called significant. These cut-off points are determined by selecting a threshold parameter “D” so that the corresponding false discovery rate (FDR) is at the desired level (e.g. 5%). In addition, fold-changes had to exceed 1.25 in order for a gene to be considered significantly up or down regulated. The SAM p-values were also determined based on the permutations. Analyses were done using an R package "samr" (http://cran.r-project.org).

Task 9. Psychosocial effects on susceptibility to SV40 large T-antigen-induced mammary cancer .

Paraffin-embedded mouse mammary gland tumor sections were assessed for tumor differentiation using a modified Bloom and Richardson System that is based on architectural features (i.e. extent of tubule formation). A total of 52 tumors from eight group-housed and 62 tumors from nine isolated mice will be evaluated. Invasive mammary adenocarcinomas showing tubular (i.e. glandular) or papillary formation in more than 75% of the tumor will be classified as grade 1 (well-differentiated). Tumors in which tubular formation constituted between 10 and 75% will be classified as grade 2 (moderately-differentiated), and tumors exhibiting less than 10% tubular formation will be classified as grade 3 (poorly-differentiated).

Task 10. Mammary gland differentiation and tumor identification.

a.) H and E analysis and histopathology diagnosis of mammary tissue differentiation and tumors.
b.) Microarray analyses of tumor tissue for receptor status: ER, PR, and GR.

Task 11. Multivariable matrix-based database established in STATA .

Develop multiple statistical models to assess interactions and relative contributions of an interdependent system, operating across levels of analysis: social, psychological, endocrine and metabolic, cellular and genetic. One possiblity is dynamic causal modeling.

a.) Transfer laboratory database and consolidate to a standardized, lab-wide, statistical program: STATA.
b.) Test hypotheses with diverse methods, multivariate modeling, log survivor analysis, analysis of variance.
c.) Identified specific functional and disease categories using IPA12 for the differentially expressed genes from isolated versus group-housed mice.

Task 12. Submit high priority manuscripts (New Specific Aim 4).

a.) Prioritize resubmitting the mammary tumor manuscript to Proceedings of the National Academy of Sciences, by using immunohistochemistry of archived tissue to diagnose tumors, and hormone receptor status.
b.) Prepare manuscript reporting SV40 large T-antigen mouse model of social isolation and increase in mammary tumor burden.

Task 13. Reinstate conventional rat laboratory (New Specific Aim 5).

a.) Continue efforts to reinstate conventional rat laboratory for standard caging, with facilities and equipment for use of carcinogens as well as detailed videography of individual and social behavior in response to everyday stressors.
b.) Ensure laboratory meets all animal, safety and environmental regulatory requirements.

Year 03

Task 14. Repeat Tasks 2 - 9 for Set 2, a second replicate of Set I above; Set1 and Set 2 should identify the same gene pathways. If they do not, we will perform gene arrays on a Set 3 to resolve any significant discrepancies.

Task 15. Resume the original and revised statement of work in our restored rat laboratory, studying social isolation and gene regulation in spontaneous and carcinogen-induced rat mammary tumors. Conduct experiments following up on the discovery that neonatal handling protects against the later development of mammary cancers.

Task 16. Publish journal articles and prepare manuscripts for submission.

APPENDIX B

12 Publications and Manuscripts

Published

1. Hermes GL, Delgado B, Tretiakova M, Cavigelli SA, Krausz T, Conzen SD, McClintock MK. Social isolation dysregulates endocrine and behavioral stress responses while increasing the malignant burden of spontaneous mammary tumors. **Proceedings of the National Academy of Sciences, USA** 106:22393- 22398, 2009. PMC2799783
2. Hermes, G.L., McClintock, M.K. Isolation and the timing of mammary gland development, gonadarche, and estropause: implications for mammary tumor burden. **Developmental Psychobiology**. 50, (4): 353-360, 2008.
3. Pyter LM, Pineros V, Galang JA, McClintock MK, Prendergast BJ. Peripheral tumors induce depressive-like behaviors and cytokine production and alter hypothalamic-pituitary-adrenal axis regulation. **Proceedings of the National Academy of Sciences** USA 106:9069-9074, 2009. PMC2689998
4. Williams JB, Pang D, Delgado B, Kocherginsky M, Tretiakova M, Krausz T, Pan D, He J, McClintock MK, Conzen SD. A model of gene-environment interaction reveals altered mammary gland gene expression and increased tumor growth following social isolation. **Cancer Prevention Research (Phila Pa)** 2:850-861, 2009.
5. Yee JR, Cavigelli SA, Delgado B, McClintock MK. Reciprocal affiliation among adolescent rats during a mild group stressor predicts mammary tumors and life span. **Psychosomatic Medicine** 70:1050-1059, 2008.

Submitted, Revision In Process

6. Volden PA, Wonder EL, Skor MN, Carmean CE, Ye H, Kocherginsky M, Eleanor Smith1, Kregel S, McClintock MK, Brady MJ, and Conzen SD. Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue. **Endocrinology**.
7. Yee JR, Cavigelli SA, Delgado B, Krausz T, Conzen SD, McClintock MK. Individual Variation In Stress-Induced Corticosterone Dynamics And Spontaneous Mammary tumor Risk. **Psychoneuroendocrinology**.
8. Yee JR and McClintock MK. Psychosocial modulation of mammary gland involution and spontaneous mammary tumor risk. **Developmental Psychobiology** .

Submitted

9. Gehlert S, Obeid E, Bollinger S, Conzen S, McClintock MK Polite B, Olopade O Pathways by Which Social Environment Affects Biology to Produce Health Disparities. Invited manuscript for **Health Affairs**

Manuscripts (Results Completed)

10. Hermes G, McClintock MK. Foraging Strategies: Development of Cognitive Strategies in a Spatial Task: Endocrine & Psychosocial Mediators. Manuscript ready for submission to **Learning and Motivation**

11. Yee JR Delgado B, Krausz T and McClintock MK. Redefining Puberty: Lobular Involution Of The Mammary Gland. **Anatomical Record**.

12. Yee JR, Delgado B, Krausz T, McClintock MK. Spontaneous Mammary Tumors In Norway Rats: A Natural Model for Human Breast Cancer and Benign Pathology. **Modern Pathology**